Polymerase δ replicates both strands after homologous recombination-dependent fork restart

Article (Accepted Version)

Polymerase δ replicates both strands after homologous recombination-dependent fork restart

Izumi Miyabe[1], Ken'Ichi Mizuno[1], Andrea Keszthelyi[1], Yasukazu Daigaku[2], Meliti Skouteri[1], Saed Mohebi[1], Thomas A. Kunkel[3], Johanne M. Murray[1] and Antony M. Carr[1,4]

[1] Genome Damage and Stability Centre, University of Sussex, Brighton, United Kingdom.

[2] Frontier Research Institute for Interdisciplinary Sciences, Tohoku University, Aoba-ku, Sendai, Japan

[3] Genome Integrity and Structural Biology Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services, Research Triangle Park, NC 27709, USA

4 Corresponding author: a.m.carr@sussex.ac.uk

Running title:

Delta:delta replication upon restart

Abstract

To maintain genetic stability DNA must be replicated only once and replication completed even when individual replication forks are inactivated. Because fork inactivation is common, the passive convergence of an adjacent fork is insufficient to rescue all inactive forks. Thus, eukaryotic cells have evolved homologous recombination-dependent mechanisms to restart persistent inactive forks. Completing DNA synthesis via Homologous Recombination Restarted Replication (HoRReR) ensures cell survival, but at a cost. One such cost is increased mutagenesis caused by HoRReR being more error prone than canonical replication. This increased error rate implies that the HoRReR mechanism is distinct from that of a canonical fork. Here we exploit the fission yeast *Schizosaccharomyces pombe* to demonstrate that a DNA sequence duplicated by HoRReR during S phase is replicated semi-conservatively, but that both the leading and lagging strands are synthesised by DNA polymerase delta.

Introduction

Accurate and complete DNA replication is prerequisite for maintaining genetic stability. Perturbations to replication underpin a range of genetic alterations, including the translocations and copy number variations typical of human genomic disorders and cancer[1-6]. The completion of DNA replication is routinely challenged by a range of replication fork barriers (RFBs) that can interfere with DNA synthesis. These include multiple forms of DNA damage, non-histone protein:DNA interactions, DNA secondary structures, clashes with the transcription machinery, DNA:RNA hybrids, programmed RFBs and DNA topology[7,8]. Replication also pauses stochastically and in response to the incorrect regulation of dNTP pools[7].

Arrested forks are initially stabilized by the intra-S phase checkpoint to allow later continuation[9]. We term such forks as "paused" and describe their subsequent continuation as "resumption". However, some arrested forks are not able to resume replication, either due to the initial nature of their arrest[1] or the stochastic failure of the intra S phase checkpoint to stabilize them. We term forks that cannot resume as "collapsed". A collapsed fork cannot, by definition, resume replication but it can be actively "restarted". In eukaryotic cells, the known mechanisms of fork restart require the homologous recombination (HR) machinery.

The preferred mechanism for dealing with an arrested replication fork is to stabilize it using the intra-S phase checkpoint, thus preventing inappropriate DNA transactions[10,11]. This stalled fork can then either resume replication when the original problem has been resolved, or await a converging fork that will merge with it and complete replication of the locus[9]. Collapsed forks cannot resume replication,

but like stalled forks that cannot bypass the original problem, they can await merger with a converging fork. To help promote such fork merging (and thus replication completion) dormant DNA replication origins are present throughout the genome and these can fire in response to local replication delays[12]. However, if a fork collapses in a region with low origin density, or at a locus where replication is unidirectional (such as a telomere, within the rDNA or proximal to a second collapsed fork) a converging fork may not be available to overcome the problem. In such circumstances, restart of the replication machinery using HR provides an additional opportunity to complete DNA synthesis[13].

Several site-specific experimental systems have been developed to characterise the mechanisms by which HR initiates DNA replication. In *Saccharomyces cerevisiae* the generation of a one-ended DSB outside of S phase has been used to initiate replication and thus model fork restart from a DSB[14]. This is defined as Recombination Dependent Replication (RDR) by break-induced replication (BIR). Experimentally, BIR is instigated outside of S phase and it takes several hours from the initial strand invasion to the onset of DNA synthesis[15]. This is likely because the recombination execution checkpoint (which usually promotes second-end capture) must first be overcome[16]. In *Schizosaccharomyces pombe* the *RTS1* replication fork barrier (RFB) has been used to initiate HR-dependent replication. Experimentally, *RTS1* generates a collapsed fork within S phase that is rapidly processed by HR proteins and initiates Homologous Recombination Restarted Replication (HoRReR) during S phase[17-19]. Importantly, while it requires the HR machinery, in the *RTS1* system HoRReR does not initiate through a DSB intermediate[17,20], formally distinguishing it from RDR by BIR.

While HoRReR allows the completion of S phase under circumstances where forks cannot resume and dormant origins are not available, there are costs associated with completing replication in this way. First there is an increase in non-allelic HR (NAHR) during the restart event itself[13,17] and second it has recently become clear that, once restarted, the resulting replication machine is error-prone. This has been characterized for RDR initiated by a DSB outside of S phase using BIR systems in *S. cerevisiae*[21-23] and for HoRReR initiated during S phase without a DSB intermediate from the *RTS1* RFB using *S. pombe*[24,25]. These increased error rates imply that RDR and HoRReR forks are non-canonical. To begin to understand the nature of a HoRReR fork, we exploited the *RTS1* RFB to explore which DNA polymerases synthesize the DNA during HoRReR.

Results

RTS1 is a ~850 bp DNA sequence that acts as a polar (i.e. direction-specific) replication fork barrier in fission yeast. *RTS1* contains several binding sites for a Myb-domain protein, Rtf1, which is necessary for fork arrest[26]. In the absence of the

rtf1^{+} gene, *RTS1* is replicated normally: there is no intrinsic feature of its sequence that is difficult to replicate and the barrier activity is entirely dependent on Rtf1 binding. To establish the nature of HoRReR following *RTS1*-induced replication arrest and HR-dependent replication restart we exploited the *T45R* construct (Fig. 1a; see methods) where *RTS1* is integrated into the genome such that a replication fork is initiated from *ars3004/3005* and proceeds towards *RTS1* where it is inactivated and subsequently restarted by HR (Fig. 1a). To minimize fork convergence from the distal side, we introduced multiple copies of TER2/3, an rDNA fork barrier. Unlike *RTS1*, TER2/3 barriers slow forks down, but do not collapse or inactivate them[25,27]. The presence of the TER2/3 barriers ensured that in 75-80% of cells the fork inactivated at *RTS1* restarts by HR and the 2.9 kb *ura5-ura4* region is replicated by HoRReR[25].

First we determined the timing of HoRReR (Fig. 1b). Cells arrested by the *cdc25-22* temperature sensitive mutation were synchronously released into the cell cycle and DNA duplication was followed by quantitative PCR using the indicated primers. Fork arrest was controlled[13] by the presence (ON) or absence (OFF) of *rtf1*$^{+}$. When the barrier was inactive, loci either side of *RTS1* replicated together. In the presence of barrier activity, duplication of the downstream loci was delayed ~20 minutes. This is consistent with data demonstrating that recombination proteins rapidly associate with *RTS1* when forks collapse[19] and that the recombination structures associated with HoRReR that can be visualized by 2D-gel analysis are resolved before mitosis[18].

Replicative polymerase usage during HoRReR

During canonical DNA replication leading and lagging strand synthesis are performed by Polε and Polδ respectively. To establish which polymerases replicate the leading and lagging strands during HoRReR, we exploited alleles (Polε; *cdc20-M630F* and Polδ; *cdc6-L591G*) that incorporate excess ribonucleotides[28,29]. DNA strands replicated by one or other of these mutant polymerases will harbor extensive rNTPs that, in the absence of RNaseH2 activity, allows the use of a strand-specific alkali liability assay to infer which polymerase replicated either the Watson or Crick strand of any specific locus[28].

We examined two regions (Fig. 1a): a control region between *ars3004/3005* and *RTS1* that should not be affected by *RTS1* activity and the experimental *ura5-ura4* region immediately downstream of *RTS1*. To determine the difference between canonical replication (barrier activity OFF) and HoRReR (barrier activity ON), strand-specific alkali sensitivity was assayed in either the absence (*rtf1*Δ) or the presence (*rft1*$^{+}$) of the Rtf1 protein cofactor. Two further control conditions were included: first, the mutant polymerases were compared to the wild-type polymerases. Second, the presence or absence of RNaseH2 (*rnh201*Δ) was matched (in the presence of RNaseH2 ribonucleotides are rapidly excised).

The Polε and Polδ mutations were analysed separately (Fig. 1c,d). As expected, in strains where the polymerases were wild-type, we did not observed high levels of alkali sensitivity for any strand. Similarly, when RNaseH2 was active, we did not observe alkali sensitivity. In the *rnh201*Δ background, the control region that is replicated canonically showed leading strand-specific alkali sensitivity in the presence of *cdc20-M630F* (Polε; Fig. 1c; c.f. lanes 20,24), irrespective of barrier activity. Similarly, we observed lagging strand-specific alkali sensitivity in the presence of *cdc6-L591G* (Polδ; Fig. 1d; c.f. lanes 28,32). Thus, *rtf1*$^{+}$ expression does not affect normal replication. In contrast, for the experimental region that is replicated by HoRReR in the presence of *rtf1*$^{+}$, in the *cdc20-M630F* (Polε) background leading strand alkali sensitivity was only observed when *RTS1* was inactive (Fig. 1c; c.f. lanes 4,8). Thus, Polε is not involved in catalyzing synthesis of either strand when DNA replication is restarted by HR. For the *cdc6-L591G* allele (Polδ), we observed lagging strand alkali sensitivity irrespective of barrier activity (Fig. 1d; c.f. 12,16). We found that the leading strand also became sensitive in the presence of *RTS1* barrier activity (Fig. 1d; c.f. lanes 4,8). Thus, both strands are synthesised by Polδ when replication is restarted by HR (HoRReR).

We have previously demonstrated that the error prone nature of HoRReR forks declined to approximately 25% over the first ~1 kb of DNA replication and then stabilized at a high level over the subsequent ~1.5 kb[25]. To eliminate the possibility that rNMPs were only incorporated into both strands over this first ~1kb we re-analysed the experimental region in two parts (Fig. 2a). We observed broadly equivalent leading and lagging strand degradation for both fragments, indicating high levels of ribonucleotide incorporation across the region.

Pol*α* initiates each DNA synthesis event and thus contributes substantially to lagging strand synthesis. To address the role of Pol*α* during HoRReR we used a genetic assay that exploits a highly mutagenic allele of Pol*α*, *swi7-H4*, that increases the mutation frequency[30] by a factor of approximately 250. Since *ura4*$^{+}$ and *ura5*$^{+}$ are the only genes in *S. pombe* that confer 5-fluroorotic acid (5FOA) resistance when inactivated by mutation, and these both reside within the experimental region replicated by HoRReR when the *RTS1* barrier is active, we used the mutation rate to 5FOA resistance as an indication of the contribution of Pol*α* to DNA synthesis of this region (Fig. 2b). When the *ura4-ura5* locus was replicated by HoRReR (barrier ON) in the presence of wild type Pol*α* (*swi7*$^{+}$) we observed that the mutation rate of the *ura4* and *ura5* at the *T45R* locus increased 4.3 times compared to when the barrier was OFF (from 1.1 to 5.2 x 10^{7}). This is consistent with previous reports that HoRReR is prone to replication slippage errors[24]. In the presence of the mutagenic *swi7-H1* (Pol*α*) allele and when the *RTS1* barrier activity was OFF (canonical replication), we observed that the mutation frequency increased by a factor of 250 when compared to *swi7*$^{+}$ cells (1.1 to 300 x 10^{7}). This reflects the substantial usage of Pol*α* during canonical replication. However, when the barrier was active in the *swi7-H1* (Pol*α*)

strain background (the *ura4* and *ura5* genes are replicated by HoRReR), we observed that the mutation rate decreased by a factor of 5.2 (300 to 58 x 10^7) when compared to the frequency seen when replication was canonical in the *swi7-H1* (Polα) background (barrier OFF). This implies that, when compared to unperturbed replication, Polα is polymerizing less DNA during HoRReR.

HoRReR forks are replicated semi-conservatively

BIR initiates replication from a DNA DSB and proceeds via a migrating D-loop[31-33]. A similar mechanism could explain the lack of Polε usage during *RTS1*-dependent HoRReR. D-loop replication is conservative, i.e. the two newly synthesised strands are present in the same sister duplex (Fig. 3a). We thus established (Fig. 3b) the mode of replication of the *ura4-ura5* locus using density substitution[34]. Arrest ON (*rtf1*$^+$) or arrest OFF (*rtf1*Δ) *cdc6-L591G* (Polδ) *rnh201*Δ cells were first synchronized in G2 and the cultures then divided into two aliquots. One aliquot from both the ON and OFF cultures was harvested immediately, representing a "light-light" isotope control (Fig. 3c). The other aliquot of each culture was transferred into media containing heavy isotope and allowed to proceed through mitosis and enter and complete S phase before harvesting. DNA was then prepared, digested with *Hind*III and *Blp*I to release the *ura4-ura5* fragment and subjected to density gradient centrifugation (Fig. 3c). At time zero, we observed that the *ura4-ura5* DNA peaked as light-light, as expected. Following either unperturbed replication (OFF), or HoRReR subsequent to fork arrest and restart (ON), we observed that the *ura4-ura5* DNA peaked as heavy-light. Importantly, no heavy-heavy DNA was detected. By subjecting an aliquot of DNA to alkali electrophoresis and probing separately for the leading and lagging strands (Fig. 3d), our alkali-sensitivity assay confirmed that the *rtf1*$^+$ (arrest ON) strain replicated the leading strand using Polδ. Thus, replication after HR-restart is semi-conservative and mechanistically distinct from BIR.

HoRReR errors are not intrinsic to δ:δ replication

HoRReR forks are prone to error. In the *RTS1* system HoRReR causes an increase in template switches at micro-homology[24] and a high rate of fork U-turn between inverted repeats[25]. One possibility is that these errors are simply a consequence of using Polδ to replicate both DNA strands. It has been previously shown that the catalytic activity of Polε is not essential for cell viability[35] and it is known that, during replication driven by the SV40 T antigen, Polδ can replicate both strands[36]. Thus, in the absence of the Polε catalytic domain it has been proposed that Polδ replicates both the leading and the lagging strand. We therefore assayed replication slippage and fork U-turn in an *S. pombe cdc20-ΔN* strain in which the catalytic domain of Polε is deleted[37]. First, we established that the bulk of DNA synthesis was indeed performed by Polδ in *cdc20-ΔN* cells. The alkali sensitivity of the previously characterized *ura4* locus was assessed in *cdc6-L591G* (Polδ) *cdc20-ΔN rnh201Δ* cells (and relevant controls) using strand-specific probes (Fig. 4a). We observed that both the leading and the lagging strands were degraded (and thus synthesised by Polδ;

see lane 4, leading and lagging strand Southern blots) when Polε was catalytically inactive, confirming its expected substitution by Polδ.

To measure the level of microhomology-mediated template exchange (replication slippage) in cells where the Polε catalytic domain is deleted we exploited the *Rura4-sd20* loci (Fig. 4b). This contains a 20 bp duplication within the *ura4* ORF which, when deleted by replication slippage, restores uracil prototrophy[24]. As previously reported, in the *cdc20*$^{+}$ (Polε^{+}) background, we found that microhomology mediated slippage at *ura4-sd20* increased by an order of magnitude when *ura4-sd20* was replicated by HoRReR forks (Fig. 4c). In the *cdc20-ΔN* (Polε catalytic deletion) background, replication without fork arrest and restart (arrest OFF) we observed an increase in slippage of ~5 times more when compared to the *cdc20*$^{+}$ (Polε^{+}) background. This demonstrates that intrinsic replication by Polδ on both strands is prone to replication slippage. However, when replication of the locus was performed by HoRReR forks (arrest ON) in the *cdc20-ΔN* background, we found that slippage increased a further factor of 2, becoming comparable to Polε^{+} arrest ON cells. This demonstrates that, while intrinsic replication by Polδ on both strands is prone to replication slippage, this error frequency is further increased when replication occurs by HoRReR and that there must therefore be additional features of the HoRReR machinery that cause these replication errors.

To assess gross chromosomal rearrangements (GCRs) caused by fork U-turn at an inverted repeat during intrinsic replication (arrest OFF) in the *cdc20-ΔN* (Polε catalytic deletion) background, we exploited the TpalR loci (Fig. 4d). This construct has a short inverted repeat downstream of the *RTS1* barrier that is highly prone to generating acentric and dicentric chromosomes when replicated by HoRReR[25]. These GCRs are detected by Southern blot: the parental chromosome is represented by a 9.9 Kb fragment, while the dicentric chromosome (the product of the U-turn) is represented by a 14.4 kb fragment. In the absence of replication arrest (*rtf1Δ*) we observed a similar low level of GCRs (Fig. 4e) when comparing *cdc20*$^{+}$ (Polε^{+}) and *cdc20ΔN* (Polε catalytic deletion). This indicates that replication of both strands by Polδ is not intrinsically prone to fork U-turn and the generation of GCRs at this locus. We note a small but reproducible increase in higher molecular weight products (greater than the 14.4 kb dicentric fragment; marked with a ? in Fig. 4e) that we are not currently able to characterise.

When replication fork arrest was induced (P_{urg1}-*rtf1*) and cells grown for approximately 3 cell cycles (Fig. 4f), we observed that the level of the rearrangement increased over the time course for both *cdc20*$^{+}$ (Polε^{+}) and *cdc20-ΔN* (Polε catalytic deletion) backgrounds. Thus, intrinsic replication by Polδ on both strands is not prone to generating GCRs at inverted repeat sequences and such rearrangements are increased in the *cdc20-ΔN* (Polε catalytic deletion) background when replication occurs by HoRReR. We attribute the elevated levels of rearrangement seen at time

zero in the *cdc20-ΔN* P_{urg1}-*rtf1* background (Fig. 4f) to an increased promoter leakiness in these cells when compared to *cdc20*$^{+}$ P_{urg1}-*rtf1*. The important point is that this elevated frequency is not seen in the *rtf1*Δ background (Fig. 4e).

Biased Polδ usage may indicate chromosome fragility

If leading and lagging strand replication by Polδ is a common feature of HoRReR, some genomic fragile sites may show a relative increase in Polδ usage for duplex DNA. This predicts that Polδ usage enriched regions in unperturbed cells could reflect intrinsic fragile sites. We have previously reported that many loci are slightly enriched for Polδ usage when compared to the genome average[29] and that the majority of these corresponded to efficient replication origins. We postulated that this may reflect occasional leading strand initiation by Polδ and the subsequent replacement of Polδ on the lagging strand by Polε as replication progresses[29].

After eliminating origin-associated regions that show a relative enrichment for Polδ usage, plus loci with heterochromatic and repetitive features such as centromeres, telomeres and the mating type locus, several Polδ-enriched regions remained that show no obvious correlative features. We chose four such regions (Fig. 5a) and, avoiding annotated features such as transcription units, integrated a 1.2kb inverted repeat at several positions, either where Polδ was relatively enriched (blue) or at unenriched flanking controls (red). When replicated by HoRReR forks the inverted repeat is highly prone to U-turn and concomitant GCR formation that can be physically assayed by quantification of Southern blots[25]. We observed that two sites (1 and 3; Fig. 5b) showed a statistically significant increase in the level of GCRs associated with Polδ enrichment when compared to the control sites ($p=0.037$ and 0.018). A third site showed a similar trend that did not reach statistical significance, while a fourth site showed no difference.

Discussion

To understand the mechanisms of genetic instability underpinning genomic disorders and carcinogenesis it is important to understand the impediments to replication, the cellular responses to these impediments and the mechanism by which these operate to ensure complete and faithful replication. Despite the function of the intra-S phase checkpoint, cells cannot avoid replication breakdown and use homologous recombination (HR) to both protect[38] and restart replication forks that are inactivated[39,40]. The nature of HR is such that it becomes a double edged sword; the ability to resume replication arises at the cost of potential NAHR[13], which causes chromosomal rearrangements. Further errors are introduced during the recombination dependent replication that ensues after fork restart[22,24,25].

The nature of HR-restarted replication

In *S. cerevisiae*, studies of the in vivo mutation spectra resulting from specific Polε and Polδ mutations with characterized in vitro substitution biases lead to a model by

which the labor of replication was divided: Polε synthesised the leading strand and Polδ the lagging strand[41]. This model received significant support from a separate study using *S. pombe* where an equivalent genetic experiment assigned Polδ to the lagging strand[28]. Because a Polε mutant that exhibited a biased mutational spectra could not be identified in *S. pombe*, a physical assay was used to assign Polε to the leading strand. This assay exploited the propensity of a specific Polε mutant to incorporate rNTPs at elevated frequency[28]. Subsequent, the model was supported by biochemical characterization of the Polδ and Polε holoenzymes in the context of the CMG helicase[42]. Finally, whole genome strand-specific profiling of rNTP incorporation in strains containing either Polε, Polδ or Pol*α* mutants that incorporated elevated levels rNTPs demonstrated that the division of labor between Polε and Polδ pertained across the entirety of both yeast genomes[29,43,44].

Canonical DNA replication is highly accurate[45], whereas RDR and HoRReR are surprisingly error prone[22,24,25]. Understanding the nature of the restarted replication machine is therefore of interest for understanding the causes of genetic instability and its contribution to human disease. However, it remains unclear how the different systems used to characterise RDR and HoRReR, mainly BIR in *S. cerevisiae* and *RTS1* in *S. pombe* respectively, are mechanistically related. During S phase fork collapse will result in both single-ended DNA DSBs (for example, when a fork encounters a nick in the template strand) and structures where the three duplexes remain connected by two regions of single stranded DNA. While these latter structures can be processed to form a single-ended DSB, this is not a requirement for HoRReR[20,39].

The *RTS1* model for HoRReR initiates without a DSB[20], occurs within S phase[18,19], initiates replication rapidly (Fig. 1), utilizes Polδ to synthesize both strands (Fig. 1), uses Pol*α* less frequently than canonical replication (Fig. 2) and proceeds via a semi conservative process (Fig. 3). Semi-conservative replication is incompatible with a D-loop migration mechanism, but the use of Polδ on both strands and the fact that this cannot explain all of the elevated replication errors observed during HoRReR (Fig. 4) strongly implies a mode of replication that is distinct from a canonical fork. The further distinctions between a canonical replication and *RTS1*-induced HoRReR remain to be determined.

In contrast to the *RTS1* system studied here, The BIR systems used to study RDR in *S. cerevisiae* initiates replication from a single-ended DSB outside of S phase and there is a significant delay to the onset of DNA synthesis[15]. Once RDR initiates, replication proceeds conservatively via a migrating D-loop[31-33] that often replicates to the end of the template molecule and does not meet an adjacent fork. Thus, the two systems are distinct, although it is unknown if this reflects differences between the two organisms, differences between RDR being initiated by a DSB as opposed to

HoRReR being initiated via a non-DSB intermediate, or if it reflects differences in initiating RDR during G2 phase as opposed to HoRReR within S phase.

Non-canonical restarted forks may contribute to genome instability

Oncogene activation, an early step in carcinogenesis, results in unbalanced replication which manifests as replication stress[46,47]. Such oncogene-induced stress provides a barrier to carcinogenesis through the activation of the DNA structure checkpoints[48]. When these checkpoints are subverted to allow cancer development, continued oncogene-induced stress is thought to underlie much of the characteristic genetic instability of cancers. The mechanisms by which oncogene activation promotes replication stress remain obscure, but low levels of dNTPs[49], increased replication origin firing[50-52], up-regulated transcription[51] and premature activation of structure-specific nucleases[53] have all been implicated.

The GCRs characterized in cancer are often associated with specific genomic regions such as common fragile sites (CFS) and early replicating fragile sites (ERFS). The innate fragility of some loci, when combined with oncogene-induced replication stress, likely results in increased abundance of replication fork collapse and – particularly in regions with a paucity of origins – this may increase reliance on replication restart and HoRReR. Because HoRReR in fission yeast results in the use of Polδ to replicate both strands, we reasoned that, in a population of cells, loci prone to occasional replication fork collapse and subsequent HoRReR may manifest as regions where ensemble mapping of replication polymerase usage on the duplex DNA reveals a bias towards Polδ when compared to Polε.

Analyzing our previously published data[29] we identified 4 sites such sites with unexplained increases in apparent Polδ usage and integrated a reporter for HoRReR signature errors. Overall, we cannot draw the direct conclusion that these sites represent previously unidentified fragile sites, but the fact that two showed a statistically significant increase in instability ($p=0.037$ and 0.018) when compared to matched control loci supports the possibility that some of the genomic instability associated with fragile sties in cancer development may result directly from forks restarted by HR within S phase that are non-canonical and thus prone to error.

Acknowledgments

This work was supported by Project Z01 ES065070 (TAK)from the Division of Intramural Research of the US National Institute of Health and Medical Research Council (UK) Grants G0801078, G1100074 plus European Research Council Grant 268788-SMI-DDR (AMC). We thank H. Masukata (Osaka University) for the *cdc20::hphMX6-Pnmt1-cdc20CTD*) strain.

Author contributions

AMC conceived the study. AMC, TAK, IM and JMM designed the experimental approach. IM,KM,SM,YD,AK and MS performed experiments and interpreted data. IM and AMC wrote the manuscript. TAK and JMM edited the manuscript.

References

1. Lambert, S. & Carr, A.M. Replication stress and genome rearrangements: lessons from yeast models. *Current opinion in genetics & development* **23**, 132-9 (2013).
2. Liu, P., Carvalho, C.M., Hastings, P.J. & Lupski, J.R. Mechanisms for recurrent and complex human genomic rearrangements. *Current opinion in genetics & development* **22**, 211-20 (2012).
3. Sun, Z. et al. Replicative mechanisms of CNV formation preferentially occur as intrachromosomal events: evidence from Potocki-Lupski duplication syndrome. *Human molecular genetics* **22**, 749-56 (2013).
4. Carvalho, C.M. et al. Replicative mechanisms for CNV formation are error prone. *Nature genetics* **45**, 1319-26 (2013).
5. Bignell, G.R. et al. Architectures of somatic genomic rearrangement in human cancer amplicons at sequence-level resolution. *Genome research* **17**, 1296-303 (2007).
6. Greenman, C. et al. Patterns of somatic mutation in human cancer genomes. *Nature* **446**, 153-8 (2007).
7. Barlow, J.H. et al. Identification of early replicating fragile sites that contribute to genome instability. *Cell* **152**, 620-32 (2013).
8. Lambert, S. & Carr, A.M. Impediments to replication fork movement: stabilisation, reactivation and genome instability. *Chromosoma* **122**, 33-45 (2013).
9. Errico, A. & Costanzo, V. Mechanisms of replication fork protection: a safeguard for genome stability. *Critical reviews in biochemistry and molecular biology* **47**, 222-35 (2012).
10. Lopes, M. et al. The DNA replication checkpoint response stabilizes stalled replication forks. *Nature* **412**, 557-61 (2001).
11. Higgs, M.R. et al. BOD1L Is Required to Suppress Deleterious Resection of Stressed Replication Forks. *Molecular cell* (2015).
12. Ge, X.Q., Jackson, D.A. & Blow, J.J. Dormant origins licensed by excess Mcm2-7 are required for human cells to survive replicative stress. *Genes & development* **21**, 3331-41 (2007).
13. Lambert, S., Watson, A., Sheedy, D.M., Martin, B. & Carr, A.M. Gross chromosomal rearrangements and elevated recombination at an inducible site-specific replication fork barrier. *Cell* **121**, 689-702 (2005).
14. Anand, R.P., Lovett, S.T. & Haber, J.E. Break-induced DNA replication. *Cold Spring Harbor perspectives in biology* **5**, a010397 (2013).

15. Jain, S. et al. A recombination execution checkpoint regulates the choice of homologous recombination pathway during DNA double-strand break repair. *Genes & development* **23**, 291-303 (2009).
16. Malkova, A. & Ira, G. Break-induced replication: functions and molecular mechanism. *Current opinion in genetics & development* **23**, 271-9 (2013).
17. Lambert, S. et al. Homologous recombination restarts blocked replication forks at the expense of genome rearrangements by template exchange. *Molecular cell* **39**, 346-59 (2010).
18. Mohebi, S., Mizuno, K., Watson, A., Carr, A.M. & Murray, J.M. Checkpoints are blind to replication restart and recombination intermediates that result in gross chromosomal rearrangements. *Nature communications* **6**, 6357 (2015).
19. Nguyen, M.O., Jalan, M., Morrow, C.A., Osman, F. & Whitby, M.C. Recombination occurs within minutes of replication blockage by RTS1 producing restarted forks that are prone to collapse. *eLife* **4**(2015).
20. Mizuno, K., Lambert, S., Baldacci, G., Murray, J.M. & Carr, A.M. Nearby inverted repeats fuse to generate acentric and dicentric palindromic chromosomes by a replication template exchange mechanism. *Genes & development* **23**, 2876-86 (2009).
21. Smith, C.E., Llorente, B. & Symington, L.S. Template switching during break-induced replication. *Nature* **447**, 102-5 (2007).
22. Deem, A. et al. Break-induced replication is highly inaccurate. *PLoS biology* **9**, e1000594 (2011).
23. Costantino, L. et al. Break-induced replication repair of damaged forks induces genomic duplications in human cells. *Science* **343**, 88-91 (2014).
24. Iraqui, I. et al. Recovery of arrested replication forks by homologous recombination is error-prone. *PLoS genetics* **8**, e1002976 (2012).
25. Mizuno, K., Miyabe, I., Schalbetter, S.A., Carr, A.M. & Murray, J.M. Recombination-restarted replication makes inverted chromosome fusions at inverted repeats. *Nature* **493**, 246-9 (2013).
26. Eydmann, T. et al. Rtf1-mediated eukaryotic site-specific replication termination. *Genetics* **180**, 27-39 (2008).
27. Calzada, A., Hodgson, B., Kanemaki, M., Bueno, A. & Labib, K. Molecular anatomy and regulation of a stable replisome at a paused eukaryotic DNA replication fork. *Genes & development* **19**, 1905-19 (2005).
28. Miyabe, I., Kunkel, T.A. & Carr, A.M. The major roles of DNA polymerases epsilon and delta at the eukaryotic replication fork are evolutionarily conserved. *PLoS genetics* **7**, e1002407 (2011).
29. Daigaku, Y. et al. A global profile of replicative polymerase usage. *Nature structural & molecular biology* **22**, 192-8 (2015).
30. Kai, M. & Wang, T.S. Checkpoint activation regulates mutagenic translesion synthesis. *Genes & development* **17**, 64-76 (2003).

31. Donnianni, R.A. & Symington, L.S. Break-induced replication occurs by conservative DNA synthesis. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 13475-80 (2013).
32. Saini, N. et al. Migrating bubble during break-induced replication drives conservative DNA synthesis. *Nature* **502**, 389-92 (2013).
33. Wilson, M.A. et al. Pif1 helicase and Poldelta promote recombination-coupled DNA synthesis via bubble migration. *Nature* **502**, 393-6 (2013).
34. Meselson, M. & Stahl, F.W. The Replication of DNA in Escherichia Coli. *Proceedings of the National Academy of Sciences of the United States of America* **44**, 671-82 (1958).
35. Kesti, T., Flick, K., Keranen, S., Syvaoja, J.E. & Wittenberg, C. DNA polymerase epsilon catalytic domains are dispensable for DNA replication, DNA repair, and cell viability. *Molecular cell* **3**, 679-85 (1999).
36. Waga, S. & Stillman, B. Anatomy of a DNA replication fork revealed by reconstitution of SV40 DNA replication in vitro. *Nature* **369**, 207-12 (1994).
37. Handa, T., Kanke, M., Takahashi, T.S., Nakagawa, T. & Masukata, H. DNA polymerization-independent functions of DNA polymerase epsilon in assembly and progression of the replisome in fission yeast. *Molecular biology of the cell* **23**, 3240-53 (2012).
38. Schlacher, K., Wu, H. & Jasin, M. A distinct replication fork protection pathway connects Fanconi anemia tumor suppressors to RAD51-BRCA1/2. *Cancer cell* **22**, 106-16 (2012).
39. Petermann, E., Orta, M.L., Issaeva, N., Schultz, N. & Helleday, T. Hydroxyurea-stalled replication forks become progressively inactivated and require two different RAD51-mediated pathways for restart and repair. *Molecular cell* **37**, 492-502 (2010).
40. Carr, A.M. & Lambert, S. Replication Stress-Induced Genome Instability: The Dark Side of Replication Maintenance by Homologous Recombination. *Journal of molecular biology* (2013).
41. Kunkel, T.A. & Burgers, P.M. Dividing the workload at a eukaryotic replication fork. *Trends in cell biology* **18**, 521-7 (2008).
42. Georgescu, R.E. et al. Mechanism of asymmetric polymerase assembly at the eukaryotic replication fork. *Nature structural & molecular biology* **21**, 664-70 (2014).
43. Reijns, M.A. et al. Lagging-strand replication shapes the mutational landscape of the genome. *Nature* **518**, 502-6 (2015).
44. Clausen, A.R. et al. Tracking replication enzymology in vivo by genome-wide mapping of ribonucleotide incorporation. *Nature structural & molecular biology* **22**, 185-91 (2015).
45. Lujan, S.A. et al. Heterogeneous polymerase fidelity and mismatch repair bias genome variation and composition. *Genome research* **24**, 1751-64 (2014).
46. Bartkova, J. et al. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature* **434**, 864-70 (2005).

47. Gorgoulis, V.G. et al. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature* **434**, 907-13 (2005).
48. Bartkova, J. et al. Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature* **444**, 633-7 (2006).
49. Bester, A.C. et al. Nucleotide deficiency promotes genomic instability in early stages of cancer development. *Cell* **145**, 435-46 (2011).
50. Dominguez-Sola, D. et al. Non-transcriptional control of DNA replication by c-Myc. *Nature* **448**, 445-51 (2007).
51. Jones, R.M. et al. Increased replication initiation and conflicts with transcription underlie Cyclin E-induced replication stress. *Oncogene* **32**, 3744-53 (2013).
52. Di Micco, R. et al. Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature* **444**, 638-42 (2006).
53. Neelsen, K.J., Zanini, I.M., Herrador, R. & Lopes, M. Oncogenes induce genotoxic stress by mitotic processing of unusual replication intermediates. *The Journal of cell biology* **200**, 699-708 (2013).

Figure legends

Figure 1. Polδ, but not Polε participates in bulk DNA synthesis after HR-restart
(**a**) Schematic representation of the construct containing *RTS1* and repetitive TER2/3 (*T45R*). The brown and black region is always replicated by a canonical fork. The red and blue region is replicated canonically when barrier activity is OFF, while it is replicated by Homologous Recombination Restarted Replication (HoRReR) when the barrier activity is active ON. (**b**) Delayed timing of DNA synthesis after HR-restart. Genomic DNA was extracted at the indicated time points from G2 arrested *cdc25-22* cells released synchronously into the cell cycle. Replication of the indicated loci, U5 and L3, was monitored by quantitative PCR. (**c**) The contribution of Polε to DNA synthesis during HoRReR. (**d**) Equivalent analysis of Polδ contribution to DNA synthesis during HoRReR.

Figure 2. Polδ usage is relatively uniform and Pol*α* usage decreased
(**a**) Polδ extends a minimum of several kb of the leading strand during homologous recombination restarted replication. The *ura5* (*Cla*I-*Blp*I) and *ura4* (*Hind*III-*Cla*I) regions were analysed separately. Genomic DNA from the strains indicated was digested, alkali treated and separated on an alkaline gel. The Watson and Crick strands were visualized following alkali treatment using single-stranded probes as indicated by colors of frames (c.f. Fig. 1a). Non-specific band; *. (**b**) Spontaneous mutation rates for indicated strains in a *swi7-H4* (Pol*α*) background. ON and OFF represent *rtf1*$^{+}$ and *rtf1-d*, respectively. Values: mean of three independent experiments, each with 11 independent cultures. Error bars: s.d.

Figure 3. HR-restarted replication is semi-conservative
(**a**) Possible models for progression of Homologous Recombination Restarted Replication (HoRReR). Top: following strand invasion, replication occurs via a migrating D-loop. The newly synthesised strand is used as a template for the "lagging" strand, resulting in conservative replication. Bottom: Following strand invasion and D-loop formation, the replication fork is reset such that both parental strands template new synthesis, resulting in semi-conservative replication. (**b**) Schematic representation of DNA fragment analysed. (**c**) Density substitution analysis of HR-restarted replication. Sample are digested DNA from *rnh201-d polδ-L591G* cells at (T0) or after 150 min incubation in a heavy medium (T150). The distribution of the *ura4-ura5* fragment after CsCl gradient centrifugation is shown. Upper panel: *rtf1*$^{+}$ (*RTS1* barrier activity ON). HH+LL indicates distribution of control DNA (mixture of heavy:heavy and light:light plasmid DNA). Lower panel: *rtf1-d* (*RTS1* barrier activity OFF). Error bars: s.d. n=3. (**d**) For density substitution experiments, the leading strand is synthesised by Polδ after HoRReR (see Fig. 1d). The leading and lagging strands of *ura4-ura5* were detected by single-stranded probes. Density substitution experiments in a *cdc6*$^{+}$ (Polδ^{+}) *rnh2*$^{+}$ background (where HoRReR cannot be verified at the time of the experiment) show equivalent results.

Figure 4. Instability resulting from homologous recombination restarted replication is not intrinsic to leading strand synthesis by Polδ
(**a**) Both leading and lagging stands are synthesised by Polδ in the Polε N-terminal deletion strain. Alkali sensitivity in the indicated strains was assessed by Southern blot using strand-specific probes. (**b**) Schematic of the *Rura4-sd20* locus for the microhomology mediated strand exchange assay. *ura4-sd20* contains a 20bp duplication flanked by microhomology and is phenotypically ura^{-} (**c**) Replication slippage at *Rura4-sd20* measured in a *cdc20*$^{+}$ (Polε^{+}) and a *cdc20-ΔN* strain where the catalytic domain of Polε is deleted. Replication forks were either not arrested (OFF: *rtf1*Δ) or arrested (ON: *rtf1*$^{+}$) at *RTS1.* Error bars; s.d. n=3, each with 11 independent cultures. (**d**) Schematic of the *TpalR* locus used for the Gross Chromosomal Rearrangement (GCR) assay. Homologous recombination restarted replication results in fork U-turn at the inverted repeat center, causing acentric and dicentric chromosome formation. (**e**) Southern blot of normal (9.9 kb) and dicentric (14.4 kb) chromosomes in three independent isolates of *TpalR cdc20*$^{+}$ (Polε^{+}) and *TpalR cdc20-ΔN* in an *rtf1*Δ background (no arrest). A low level of uncharacterized higher molecular weight species is evident in *cdc20-ΔN* cells (vertical bar: ?). Right: quantification of 14.4 kb (GCR) band, or all high molecular weight species. Error bars; s.d. n=3 (**f**) Southern blot of normal (9.9 kb) and dicentric (14.4 kb) chromosomes at T= 0, 3.5 and 7 hours after induction of *rtf1*$^{+}$. Right: quantification of three experiments. Error bars: s.d. The increased GCRs seen in panel f at T=0 for *cdc20-ΔN* likely represents increased leakiness of the P_{urg1} promoter used.

Figure 5. Polδ usage correlates with fragile sites

(**a**) The positions at which a the small inverted repeat (top) was integrated. Theses correspond to sites around four euchromatic regions that are not associated with a replication origin (open circles), but which showed an intrinsic relative increase in Polδ usage (Polδ:Polε ratio) for the duplex DNA. Blue: relatively increased Polδ, red: relatively increased Polε. Numbers: kb on indicated chromosome (**b**). Gross Chromosomal Rearrangements (GCRs) resulting from a fork U-turn at the center of the inverted repeat were assayed. Top: a representative Southern blot. Bottom: the % of GCRs (dicentric + acentric as a proportion of total signal) in the population quantified in 3 independent experiments. * = significant *p*-values (two-tailed Student's t test): region 1, I-3870/I-3920, 0.037 and I-3890/I-3920, 0.018. Region 3, II-1165/II-1190, 0.010.

Online Materials and Methods

S. pombe Methods

*C*ells were grown in Yeast Extract (YE) or EMM2 minimal medium. Standard genetic and molecular procedures were employed as described previously[54]. The *cdc20-ΔN* (*cdc20::hphMX6-Pnmt1-cdc20CTD*) strain[37] was grown in EMM2 without thiamine.

Construction of TER2/3-*ura4*/5-*RTS1* (T45R)

The previously described TpalR (TER2/3-*ura4* palindrome-RTS1) construct[25] was modified as below. The centromere-proximal *ura4* gene in TpalR was replaced with *Ase*I-*Hind*III fragment containing *ura5* gene. To increase the ratio of restarted forks, 10 copies of TER2/3 were inserted ~500 bp telomere-proximal of TER2/3 in the construct by recombination-mediated cassette exchange (RMCE)[55]. The original *ura5* gene on chromosome II was deleted.

Detection of alkali-sensitive sites in genomic DNA

Genomic DNA, digested with appropriate restriction enzymes, was incubated in 0.3N KOH at 55°C for 2 hours and subjected to 1% alkaline agarose gel electrophoresis. Each strand of *ura4-ura5* fragment was detected by Southern blot using a single-stranded probe as described previously[28]. All uncropped blots are shown in supplementary dataset 1.

Monitoring replication timing

cdc25-22 cells were arrested at G2 at 36°C for 3.5 hours and released into cell cycle at 25°C. Cells were collected every 15 minutes and genomic DNA was extracted. Replication was monitored by quantitative PCR (qPCR).

Determination of spontaneous mutation rates

Uracil prototrophic cells were spread on YEA (YE agar) plates and incubated at 30°C for 3 days. 11 independent single colonies were inoculated in 5ml YE and grown to saturated phase at 30°C. Cells were diluted appropriately and plated on YEA or YEA

containing 0.1% 5-fluoroorotic acid (5-FOA). Colonies were counted after 4 days incubation at 30°C. Mutation rates were calculated by the method of median[56].

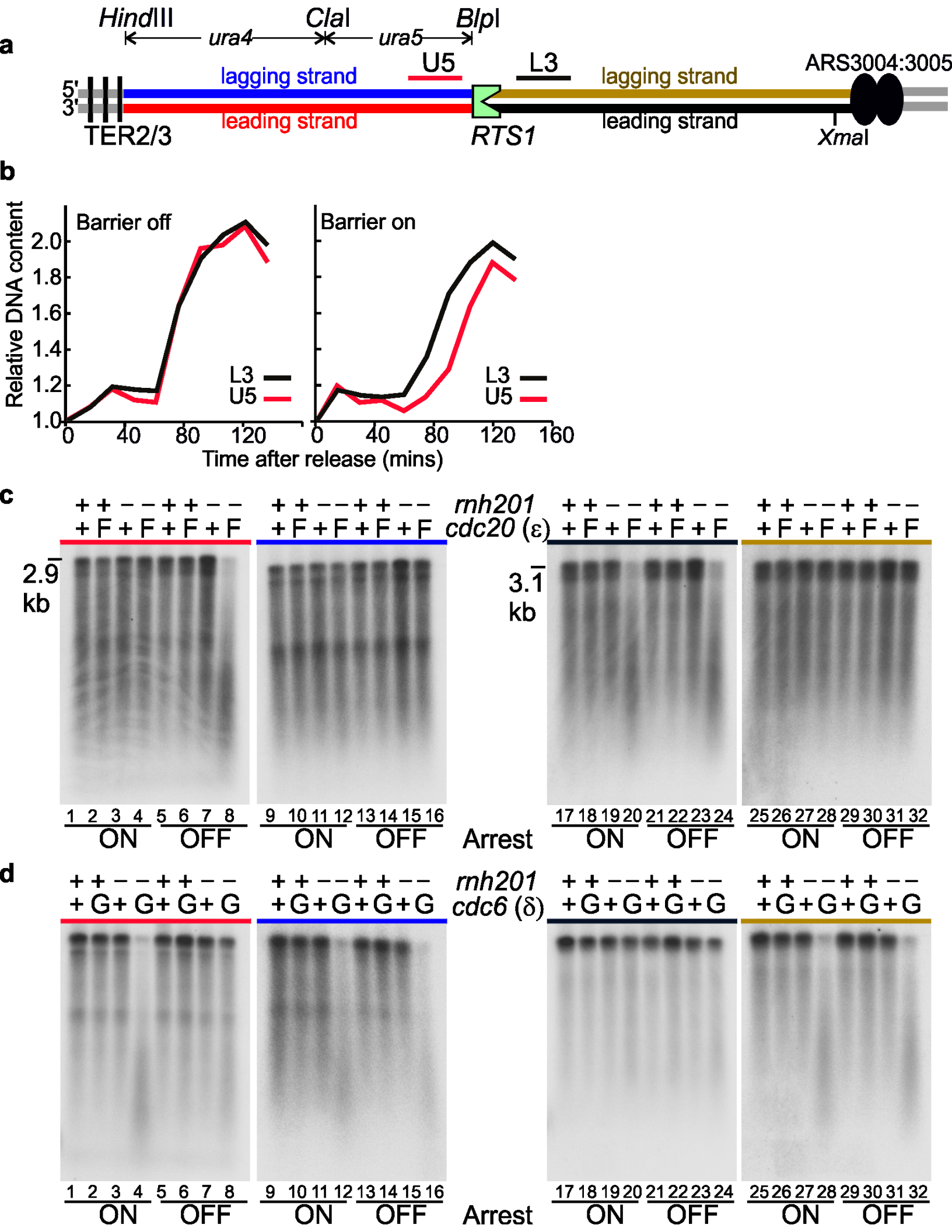
Detection of chromosomal rearrangements.
For P_{urg1}-*rtf1*$^+$ expression[57], cells were grown to log phase in EMM2 without uracil, then 250 μg/ml of uracil was added to induce expression of the *rtf1*$^+$ gene. Genomic DNA was prepared in agarose plugs, digested with an appropriate restriction enzyme after equilibration with the reaction buffer and subjected to agarose gel electrophoresis. Chromosomal rearrangements were detected by Southern blot using an appropriate probe. Band intensities were quantified using ImageJ. All uncropped blots are shown in supplementary dataset 1.

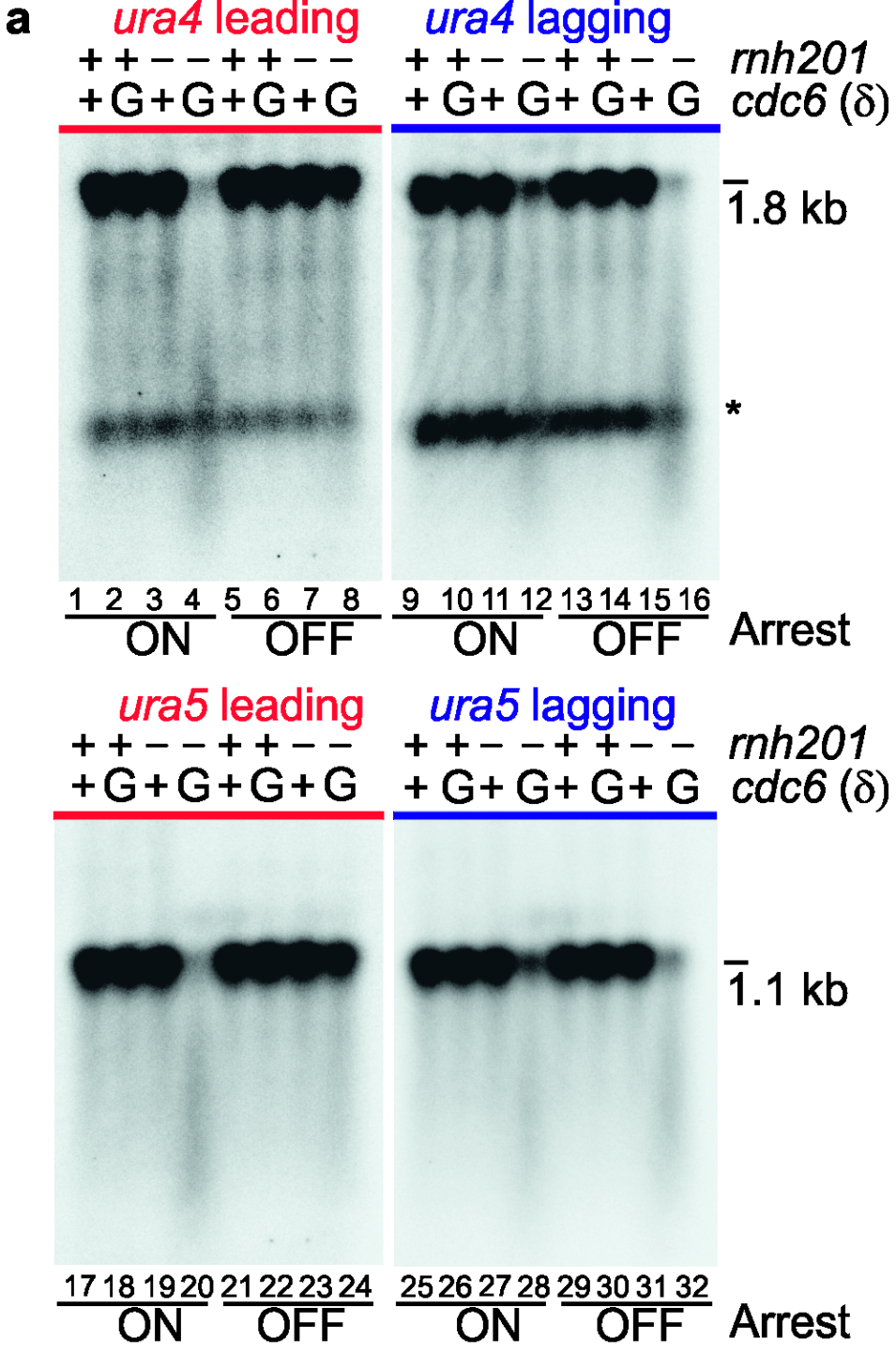
Replication slippage assay using *ura4-sd20*.
The ura$^+$ reversion assay using *ura4-sd20* was performed as described previously[24] with some modifications. Briefly, 5-FOA resistant cells were grown in uracil-containing media to saturation. Cells were washed, appropriately diluted and plated on minimal medium plates with or without uracil. Colonies were counted after 4-5 days incubation at 30°C to determine the reversion frequency.

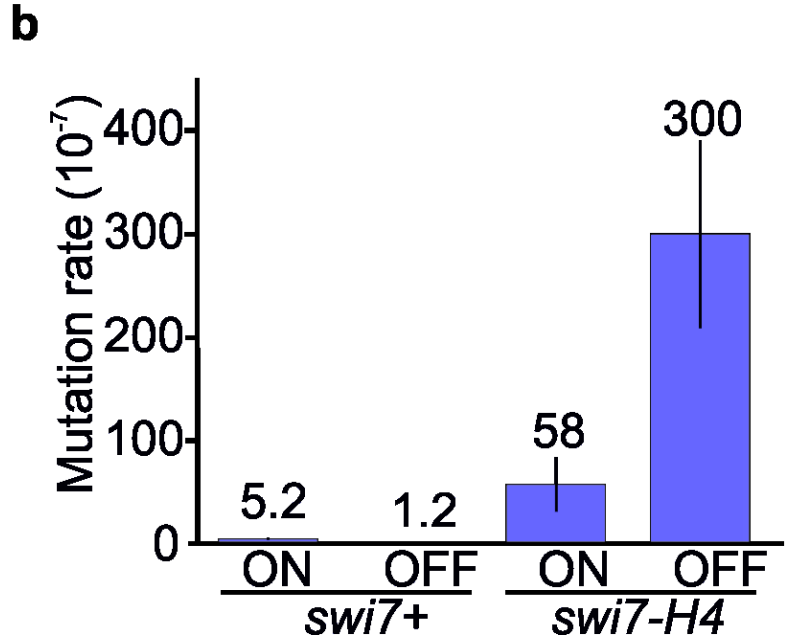
Determination of the mode of replication by density substitution
Cells were grown, synchronized and treated as described in[58]. Briefly: *rnh201-d polδ-L591G* cells were grown in EMM2[54] containing 0.2% NH_4Cl and 1% glucose were harvested and loaded on lactose gradients. After centrifugation, small (G2) cells were collected and split into two aliquots. One was centrifuged immediately (T0), the other was washed and transferred into "heavy" EMM2 containing ^{15}N NH_4Cl and ^{13}C glucose. Cells were harvested after 150 min incubation at 30°C (T150). Genomic DNA was extracted and digested with *Hind*III and *Blp*I and split into two aliquots. One was treated with alkali as described above, followed by alkaline gel electrophoresis. The other was loaded on a CsCl solution gradient and density gradient centrifugation[34] was performed at 30,000 rpm for 67 hours. The density gradient was fractionated and distribution of the *ura4-ura5* fragment was quantified by slot-blot using *ura5* probe. Relative intensity of each fraction to the peak in T0 was plotted.

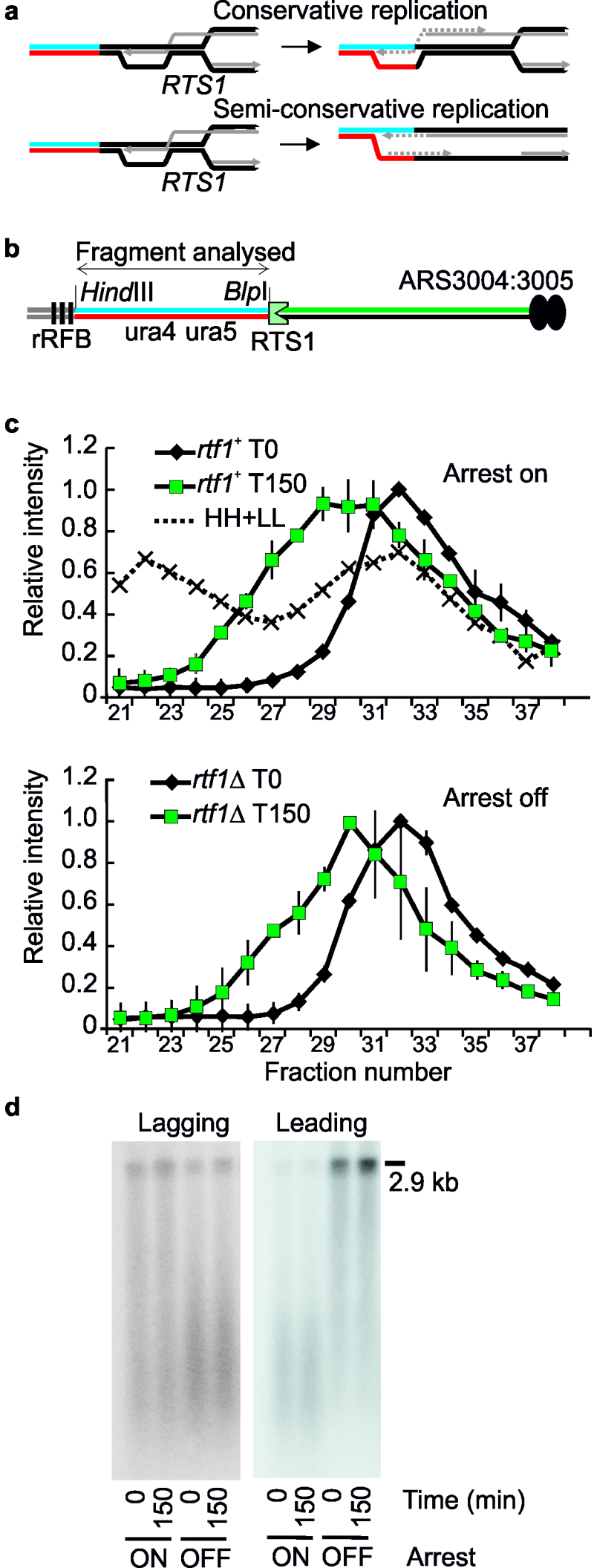
References for Online Methods
54. Moreno, S., Klar, A. & Nurse, P. Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. Methods in enzymology 194, 795-823 (1991).
55. Watson, A.T., Garcia, V., Bone, N., Carr, A.M. & Armstrong, J. Gene tagging and gene replacement using recombinase-mediated cassette exchange in Schizosaccharomyces pombe. Gene 407, 63-74 (2008).
56. Lea, D.E. & Coulson, C.A. The distribution of the numbers of mutants in bacterial populations. Journal of genetics 49, 264-85 (1949).

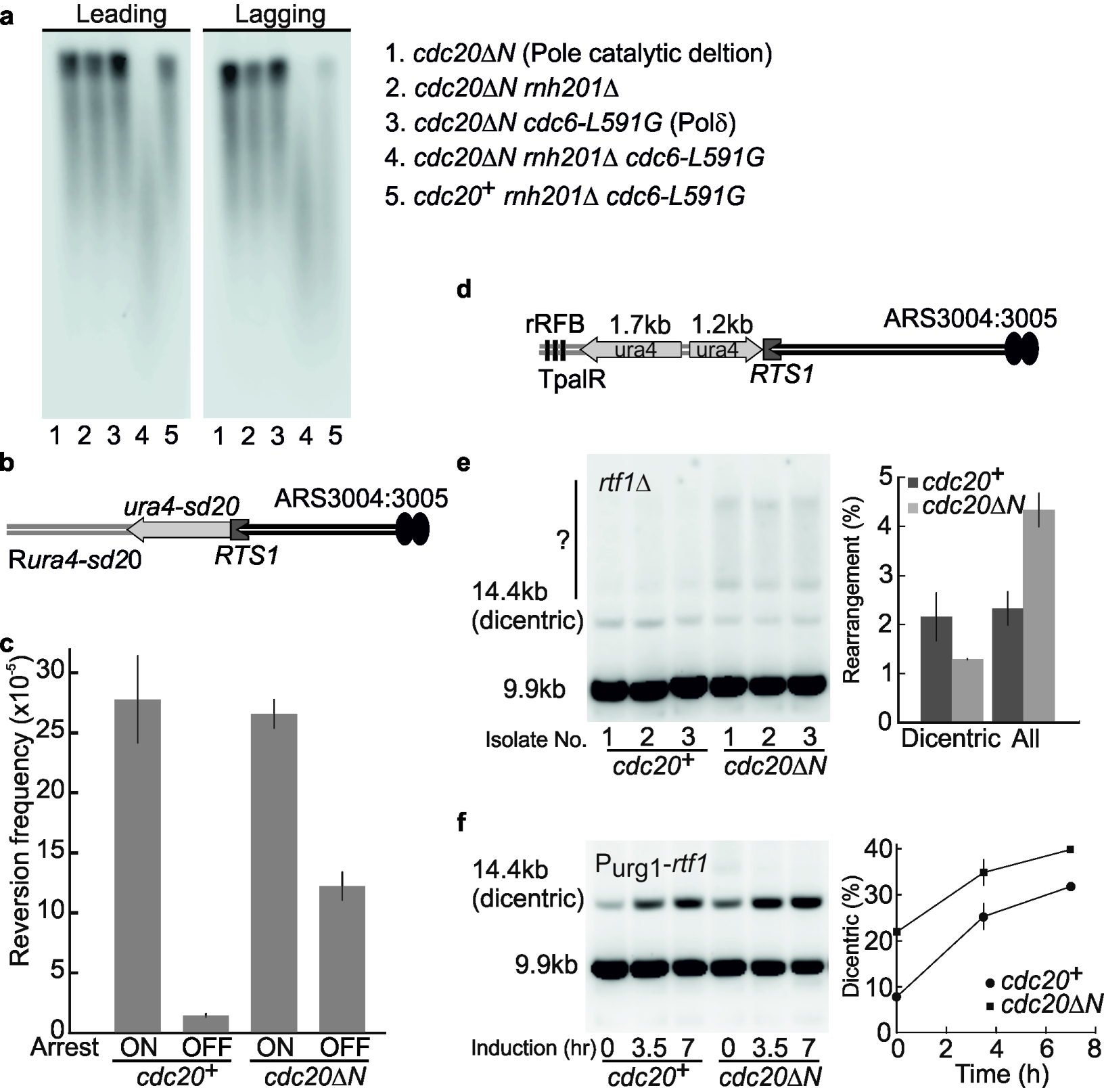
57. Watson, A.T. et al. Optimisation of the Schizosaccharomyces pombe urg1 expression system. PloS one 8, e83800 (2013).
58. Arcangioli, B. Fate of mat1 DNA strands during mating-type switching in fission yeast. EMBO reports 1, 145-50 (2000).

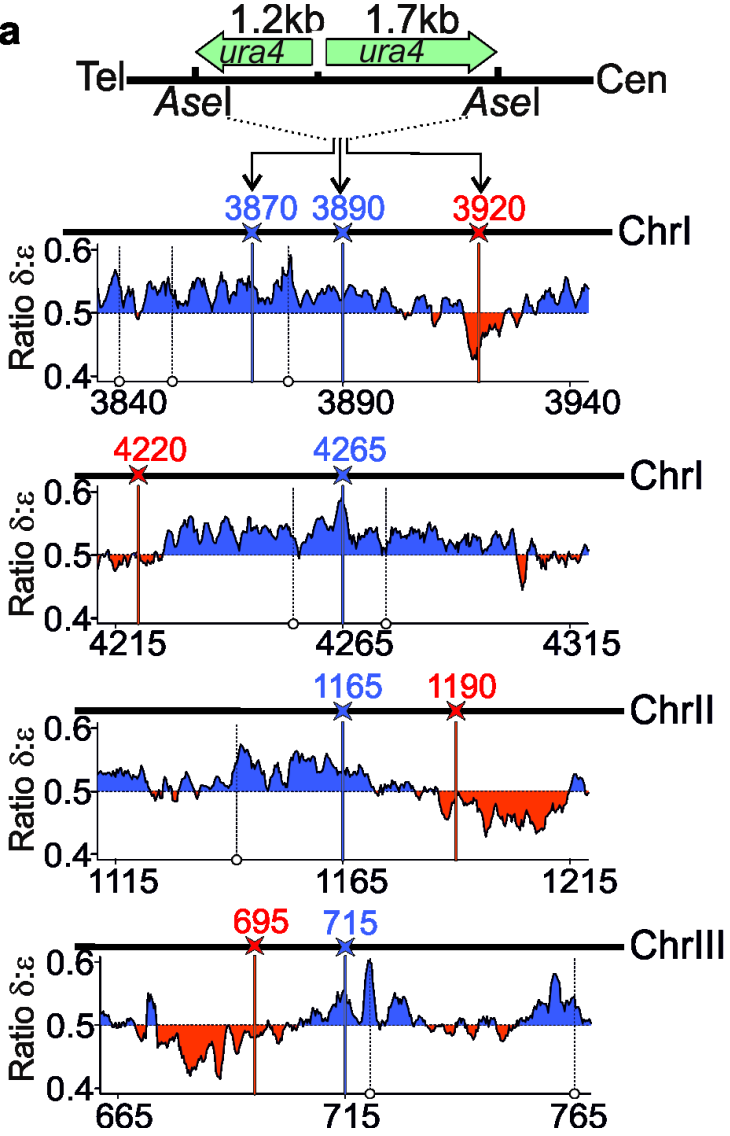

a
HindIII
ClaI
BlpI
ura4
ura5
U5
L3
lagging strand
leading strand
lagging strand
leading strand
5'
3'
TER2/3
RTS1
ARS3004:3005
XmaI
b
Barrier off
Barrier on
Relative DNA content
2.0
1.8
1.6
1.4
1.2
1.0
L3
U5
0
40
80
120
160
Time after release (mins)
c
rnh201
cdc20 (ε)
2.9 kb
3.1 kb
+ + − − + + − −
+F+F+F+F
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16
17 18 19 20 21 22 23 24
25 26 27 28 29 30 31 32
ON
OFF
Arrest
d
rnh201
cdc6 (δ)
+G+G+G+G

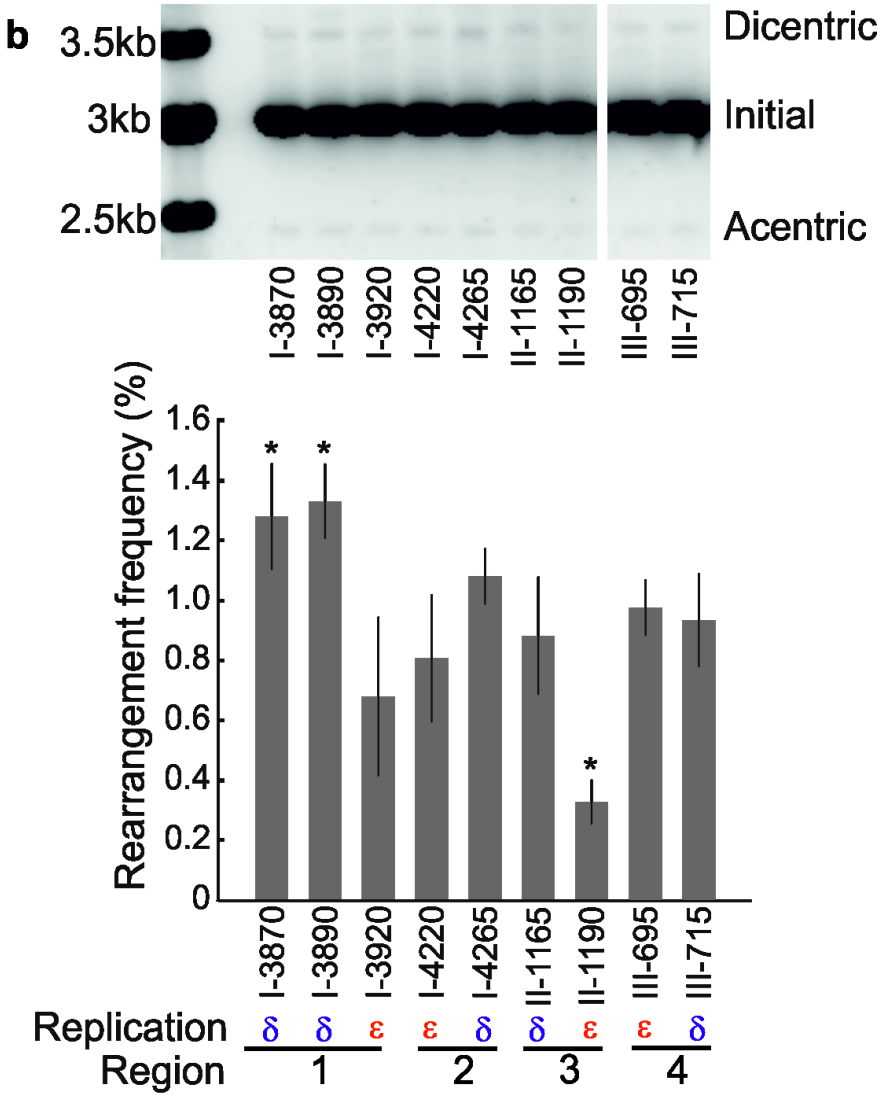

a
ura4 leading
ura4 lagging
+ + − − + + − −
+G+G+G+G
+ + − − + + − −
+ G+ G+ G+ G
rnh201
cdc6 (δ)
1.8 kb
*
1 2 3 4 5 6 7 8
ON OFF
9 10 11 12 13 14 15 16
ON OFF
Arrest
ura5 leading
ura5 lagging
+ + − − + + − −
+G+G+G+G
+ + − − + + − −
+ G+ G+ G+ G
rnh201
cdc6 (δ)
1.1 kb
17 18 19 20 21 22 23 24
ON OFF
25 26 27 28 29 30 31 32
ON OFF
Arrest


b
Mutation rate (10-7)
400
300
200
100
0
5.2
1.2
58
300
ON
OFF
ON
OFF
swi7+
swi7-H4


a
Conservative replication
RTS1
Semi-conservative replication
RTS1
b
Fragment analysed
HindIII
BlpI
ARS3004:3005
rRFB
ura4 ura5
RTS1
c
Relative intensity
rtf1+ T0
rtf1+ T150
HH+LL
Arrest on
rtf1Δ T0
rtf1Δ T150
Arrest off
Fraction number
d
Lagging
Leading
2.9 kb
Time (min)
ON OFF ON OFF
Arrest


a
Leading
Lagging
1 2 3 4 5
1 2 3 4 5
1. cdc20ΔN (Pole catalytic deltion)
2. cdc20ΔN rnh201Δ
3. cdc20ΔN cdc6-L591G (Polδ)
4. cdc20ΔN rnh201Δ cdc6-L591G
5. cdc20+ rnh201Δ cdc6-L591G
b
ura4-sd20
ARS3004:3005
Rura4-sd20
RTS1
c
Reversion frequency (x10-5)
30
25
20
15
10
5
0
Arrest ON OFF ON OFF
cdc20+
cdc20ΔN
d
rRFB
1.7kb
1.2kb
ura4
ura4
ARS3004:3005
TpalR
RTS1
e
rtf1Δ
?
14.4kb
(dicentric)
9.9kb
Isolate No. 1 2 3 1 2 3
cdc20+
cdc20ΔN
Rearrangement (%)
5
4
3
2
1
0
cdc20+
cdc20ΔN
Dicentric All
f
14.4kb
(dicentric)
9.9kb
Purg1-rtf1
Induction (hr) 0 3.5 7 0 3.5 7
cdc20+
cdc20ΔN
Dicentric (%)
40
30
20
10
0
cdc20+
cdc20ΔN
0 2 4 6 8
Time (h)


a
1.2kb
1.7kb
ura4
ura4
Tel
Cen
AseI
AseI
3870
3890
3920
ChrI
Ratio δ:ε
0.6
0.5
0.4
3840
3890
3940
4220
4265
ChrI
4215
4265
4315
1165
1190
ChrII
1115
1165
1215
695
715
ChrIII
665
715
765


b
3.5kb
3kb
2.5kb
Dicentric
Initial
Acentric
I-3870
I-3890
I-3920
I-4220
I-4265
II-1165
II-1190
III-695
III-715
Rearrangement frequency (%)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
*
*
Replication
δ δ ε ε δ δ ε ε δ
Region
1
2
3
4